METHODS

HPLC of Plasmalogen-Containing Phosphatidylcholine Under Reverse-Phase or Argentation Conditions

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ABSTRACT

Two approaches to the high pressure liquid chromatography (HPLC) isolation of intact plasmalogens were investigated. The first used reversed-phase HPLC and sought to take advantage of subtle differences in the hydrophobicity of the alk-1-enyl chain from the acyl counterpart. On a C-18 column, bovine heart phosphatidylcholine (PC), which was 47% plasmalogen, was separated into a number of fractions that differed in their molecular species composition. One combination of fractions amounted to a 26% yield of PC enriched to 82% plasmalogen. The second approach sought to take advantage of the uniquely electron-rich functionality of the plasmalogens, the alk-1-enyl ether double bond, and its potential to coordinate with heavy metal ions. Specifically, bovine heart PC was applied to a cation-exchange type HPLC column in the silver ion mode. Although complete exchange of all the active sites of the column with silver ion led to complete retention of PC, partial activation with silver ion resulted in the separation of the PC into fractions, according to the degree of unsaturation. Plasmalogen-rich fractions eluted last and remained intact during the process. One combination of these fractions amounted to a 49% yield of PC enriched to 72% plasmalogen. Use of a cation-exchange system in the mercuric ion mode led to on-column hydrolysis of the plasmalogen; with palladium ion, the metallic species was stripped from the column by the eluting lipid.
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INTRODUCTION

Chromatographic separation of intact plasmalogens (alk-1-enyl phosphatides) has eluded researchers for years. The behavior of these species during high performance liquid chromatography (HPLC) has been ignored, mainly because HPLC studies have largely been confined to model diacylphosphatides or to natural phosphatides with little or no plasmalogen content (e.g., liver, egg yolk or soy phosphatidylcholine [PC]). The plasmalogen content of natural phosphatides from muscle or heart tissue is considerable, however. The phosphatide selected for the present investigation, bovine heart PC, is ca. half plasmalogen.

The alk-1-enyl group is a unique and highly electron-rich double bond, which is easily protonated and capable of complexing with electrophilic species (such as tetracyanoethylene [1], maleic anhydride [2] and mercuric ion [3]), to a degree unseen with isolated double bonds. With the exception of early argentation thin layer chromatography (TLC) work discussed below, however, such interactions have not been used to attempt the isolation of intact plasmalogen from nonplasmalogen analogs. Typically, the chromatographic behavior of plasmalogen is governed by the polar character of the phosphobase group that it has in common with its nonplasmalogen counterparts. Successful chromatographic separations, therefore, have been achieved by first removing the polar group by enzymatic hydrolysis (4) or chemical reduction (5), or by conversion of the polar group to a nonpolar derivative (6,7). The chromatography that ensues thus ceases to be a separation of intact plasmalogen. These types of chromatographic analyses have been reviewed (8).

Successful separation of molecular species of intact phosphatides has been achieved by argentation TLC. Fractionation was based on unsaturation content, despite the common polar group shared by all the species. Although most reports dealt with phosphatides of negligible plasmalogen content (9-13), 2 studies dealt with plasmalogen-rich bovine heart phosphatides–PC (14) and phosphatidylethanolamine (PE) (15). These studies demonstrated that the lipids could be fractionated into overlapping bands on Silica Gel G plates that had been predeveloped with saturated silver nitrate. The overall separations seemed to be a function of the total degree of unsaturation, though some of the bands were found to be enriched in plasmalogen.

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Argentation HPLC of phosphatides, on the other hand, has been virtually ignored. It has been used to supplement reversed-phase HPLC (RP-HPLC) to isolate molecular species of sphingomyelin (SM) (16), whereby the argentation technique enabled the separation of critical pairs unseparable on reversed-phase columns but differing in unsaturation content. Similarly separated were critical pairs of egg-yolk PC (17).

On a different tack, another group recently has reported on the successful separation of molecular species of (plasmalogen-free) rat-liver phosphatides by RP-HPLC (18). Under these conditions, too, the influence of the polar group is minimized, this time by the dominance of hydrophobic interactions.

The present research was done to investigate the behavior of plasmalogen-rich (47 mol %) bovine heart PC when subjected to argentation and other charge-transfer HPLC, and to RP-HPLC.

EXPERIMENTAL

HPLC Instrumentation

Chromatography was run on a Beckman Model 334 Gradient Liquid Chromatograph, consisting of a Model 210 sample injection valve, a Model 421 system controller and Model 110A pumps (Beckman Scientific Instruments Division, SmithKline-Beckman, Inc., Berkeley, CA), and supplemented by an LKB Model 238 Uvicord S UV detector (LKB Instruments, Inc., Gaithersburg, MD) and an MFE Model 2125B dual pen recorder (Allen Data Graph, Inc., Salem, NH). Monitoring of column effluent was done at 206 nm.

Two strong cation-exchange columns were used; initial runs were on a Nucleosil 5 SA column, 4.0 mm i.d. × 20 cm, 5 micron particle size (Macherey-Nagel GmbH, Düren, FRG and Rainin Instruments, Inc., Woburn, MA); later runs were on a Chromegabond P-SCX column, 4.6 mm i.d. × 30 cm, 10 micron particle size (ES Industries, Marlton, NJ). For RP-HPLC, an Ultrasphere ODS column was used, 4.6 mm i.d. × 25 cm, 5 micron particle size (Beckman).

Other Instrumentation

Gas chromatographic (GC) analysis of fatty acid methyl esters (FAME) and aldehydes was performed on a Hewlett-Packard Model 5880A level 4 flame ionization capillary gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a 100 m 0.25 mm i.d. SP 2340 glass column (Quadrex, New Haven, CT). Identification of FAME was aided by standards reported in an earlier publication (19). Additionally, structural identification of individual aldehydes and FAME was confirmed by GC-mass spectrometry (MS), using a Hewlett-Packard Model 5995 instrument equipped with a Hewlett-Packard 15 m OV-101 fused silica capillary column (Hewlett-Packard, Avondale, PA); the oven was programmed from 150 to 220 C at 8 C/min.

HPLC Conditions

Cation-exchange columns were treated with silver nitrate as follows: the column first was converted from the acid mode to the sodium mode by treatment with 0.1 N $NaNO_3$ until no further detection of eluted acid (pH paper) occurred. This was followed by flushing with doubly distilled water. Then, after switching to the solvent system to be used (aqueous methanol), 0.1 N aqueous $AgNO_3$ was introduced incrementally (by 10 μl injections) to establish optimum column load. After each incremental addition, the column was evaluated with the lipid solution (20 μl of bovine heart PC in chloroform, 10 mg/ml [Sigma Chemical Co., St. Louis, MO]). Column capacity was determined by charging the column with 0.1 N HNO_3, flushing with water, collecting the effluent during charging with 0.1 N $NaNO_3$ and finally titrating the released acid with 0.1 N NaOH. In a manner analogous to charging with silver ion, the same procedure was used to incorporate palladium II ion (0.1 M $PdCl_2$ in methanol), mercuric ion (using 0.1 M aqueous mercuric acetate) and phenylmercuric ion (using 0.1 M phenylmercuric acetate in 10% aqueous methanol). (Warning: handle toxic mercuric compounds with care.)

Run conditions were as follows (solvent A = water, B = methanol): Macherey-Nagel column in Ag mode–flow, 1 ml/min; solvent gradient = 100% B to 70% B over 15 min, then isocratic; ES column in Ag mode–flow, 2 ml/min through 8 min, then to 4 ml/min over 6 min, then constant flow at 4 ml/min; solvent = 85% B; Macherey-Nagel column in Hg mode–flow, 1 ml/min; solvent = 80% B; ES column in palladium mode–flow = 1 ml/min; solvent = 80 or 85% B; Beckman reversed-phase column–flow = 2.5 ml/min; solvent = B/A/acetonitrile 95:4:1 0.13 M in choline chloride (a modification of the method of Patton et al. [18]).

Highly unsaturated PC fractions that failed to elute from the charge-transfer columns by the solvents of choice were released by injection of 80 μl of 1-hexene at the end of each run, in accordance with published results on medium pressure liquid chromatography of neutral lipids (20,21).

Metal ion leakage from the columns was detected as follows: Ag—white precipitate with aqueous NaCl; Hg and Pd—purple color with s-diphenylcarbazone (Fisher, Fairlawn, NJ).

Analysis of Lipid Fractions

Lipid fractions were analyzed for plasmalogen content by acid cleavage of the plasmalogen to aldehyde and 2-acylglycerophosphocholine, TLC to separate the fragments from unreacted nonplasmalogen PC and elemental phosphorus analysis (22) of the 2 phosphorus-containing species, according to the Horrocks procedure (23).

Aldehydes that were released from plasmalogen by acid treatment were analyzed directly by GC or converted to FAME after a mild oxidation during a multistep TLC procedure as follows: a lipid spot at the origin of a Silica Gel G TLC plate (5 x 20 cm, 250 m, Analtech, Newark, DE) was first treated with HCl vapor (23). The plate then was developed 7 cm using hexane/ether (80:20, v/v). Phosphorus-containing species remained at the origin. The aldehyde spot, which was just below the solvent front and located by spraying a parallel spot with fuchsin/bisulfite, was sprayed with a saturated solution of ceric sulfate in 0.5 N H_2SO_4. The plate was warmed for a minute on a hot plate (at the lowest setting). The cooled plate was redeveloped 14 cm in the same solvent mixture to separate any unreacted aldehyde (high R_f) from the product fatty acid (which only moved slightly from the region sprayed with ceric sulfate). The fatty acid spot was visualized under UV light after spraying with 1,6-diphenylhexatriene. Redevelopment in hexane removed the visualization reagent but left the fatty acid at its former position. The fatty acid spot was removed by scraping, eluted from the silica gel by ether and derivatized to methyl ester by treatment with diazomethane. Tests with standard saturated and monounsaturated aldehydes confirmed the validity of the oxidation, and FAME profiles from oxidation of the aldehydes released from the bovine heart PC closely resembled the profiles of the underivatized aldehydes. Finally, the phosphorus-containing species—still at the origin—could be separated from one another by developing the plate 7 cm with chloroform/methanol/water (65:25:4, v/v/v). The resulting spots could then be visualized by Phospray (Supelco, Bellefonte, PA), scraped and analyzed for phosphorus to determine the plasmalogen content of the original lipid fraction.

Acyl groups were determined by KOH/methanol derivatization to FAME by a microcolumn technique (24), followed by GC analysis (19).

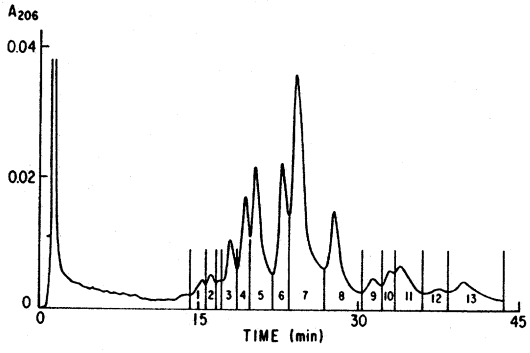

FIG. 1. RP-HPLC of bovine heart PC (0.26 μmol). Column: Beckman Ultrasphere ODS; conditions: cf. Experimental; peak numbers correspond to fraction numbers in Table 1.

TABLE 1

Lipid Analysis of Bovine Heart PC Fractions Separated by RP-HPLC

Fraction (peak number[a])	Recovered PC[b] (%)	Plasmalogen content[c] (%)	Fraction (peak number)	Recovered PC (%)	Plasmalogen content (%)
1	1.2	5.6	8	18.1	31
2	5.6	33	9	7.3	45
3	4.3	41	10	4.3	18
4	5.2	44	11	6.5	74
5	15.7	15	12	2.5	54
6	7.2	76	13	3.6	52
7	18.4	85	Total	100	47

[a]Cf. Figure 1.

[b]Mol percentage of total PC, based on phosphorus analysis.

[c]Mol percentage of fraction, based on phosphorus analysis (cf. Experimental).

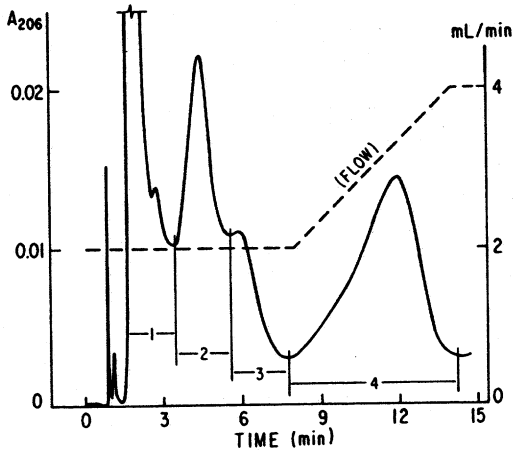

FIG. 2. Argentation HPLC of bovine heart PC (0.26 μmol). Column: ES Chromegabond P-SCX; Ag/PC = 86:1; other conditions—cf. Experimental; peak numbers correspond to fraction numbers in Tables 2 and 3.

RESULTS AND DISCUSSION

Figure 1 shows the trace from a reversed-phase separation of bovine heart PC. Table 1 provides the information on lipid analysis of the components labeled in this figure.

Although the pattern of plasmalogen elution from this column was unpredictable, the fraction giving the largest detector response (peak 7) was highly enriched in plasmalogen content. Together with the preceding fraction (peak 6), RP-HPLC allowed 26% of the total PC to be isolated with an 82% plasmalogen content.

TABLE 2

Lipid Analysis of Bovine Heart PC Fractions from Argentation HPLC[a]

Fraction[b]	Recovered PC[c] (%)	Plasmalogen content[d] (%)
1	10	19
2	41	19
3	8	78
4	30	75
5 (postwash)	11	58
Total	100	47

[a]Run on ES column.

[b]Cf. Figure 2.

[c]Mol percentage of total PC, based on phosphorus analysis.

[d]Mol percentage of fraction, based on phosphorus analysis (cf. Experimental).

Because the present research sought a chromatographic procedure that allowed isolation of plasmalogen by virtue of the unique alk-1-enyl ether functionality of that class of lipids, greater attention was given to charge-transfer HPLC than to RP-HPLC. The ideal electrophile for HPLC would have to interact specifically with the alk-1-enyl double bond, but only in a reversible way, and without catalyzing any reaction of the double bond with the mobile phase. The electrophile would best be immobilized on a strong cation exchange column. Initial experiments with such argentation-HPLC showed that bovine heart PC could be fractionated on a 5-micron column (Macherey-Nagel) into 4 major fractions. This separation

TABLE 3

Moles of Acyl and Alk-1-enyl Chain per 50 Mol of Bovine Heart PC[a]

C=C per chain: Type of chain:	0 acyl	1 acyl	1 alkenyl	2 acyl	3 acyl	4 acyl	5 acyl	Total chain	Average C=C per chain
Fraction									
Total PC	28.9	16.8	23.5	21.6	3.3	4.9	1.1	100	1.2
Plasmalogen	1.7	6.1	23.5	11.4	1.7	2.3	0.5	47	1.5
Other PC	26.3	13.6	0.0	10.3	1.3	1.3	0.2	53	0.8
Sum	28.0	19.7	23.5	21.6	2.9	3.6	0.7	100	1.1
1	5.1	3.9	1.0	0.1	0	0	0	10	0.5
2	15.0	8.6	3.9	13.4	0	0	0	41	1.0
3	1.3	2.5	3.1	0.7	0.1	0.2	0	8	1.1
4	2.0	1.1	11.3	12.6	2.1	0.9	0	30	1.6
5	1.3	0.2	3.2	0.5	1.0	4.2	0.7	11	2.5
Sum	24.7	16.4	22.4	27.3	3.2	5.3	0.7	100	1.3

[a]On ES column.

resulted from adjusting the silver ion load to a silver/lipid ratio (Ag/PC) of 86:1. (PC did not elute from a column that had been saturated with silver ion [Ag/PC = 4000:1].) TLC analysis showed that each fraction was progressively more enriched in plasmalogen content. Nevertheless, very high column pressures (ca. 4,000 lb/sq in.) were necessary. Therefore, this column, with particle size of 5 microns, was replaced with another one (ES) of 10-micron particle size. The optimized separation on this ES column still was achieved with Ag/PC = 86:1, though the elution pattern shown in Figure 2 differed from the pattern seen from the earlier column. Required adjustments of solvent composition and flow rate, and the differences in elution patterns from column to column no doubt reflected nonuniform distribution of silver ion during partial argentation of an existing column. Better consistency may be possible by packing a column with support that already has been partially argentated. However, this would require testing several columns that differ in their silver load. Table 2 gives the lipid analysis for the separation shown in Figure 2. The initially eluting fractions (1 and 2) were low in plasmalogen content, whereas the later ones (3-5) were rich in it. Fraction 5 was the postwash, the lipid that was released by treatment with 1-hexene.

To understand the processes that led to plasmalogen enrichment of the later fractions, the recovered lipids were analyzed for their acyl and alk-1-enyl content. Direct GC analysis of aldehydes liberated from the fractions showed essentially no difference in aldehyde composition from fraction to fraction. Oxidation and derivatization to FAME showed that the aldehydes were mainly palmitaldehyde with some stearaldehyde and olealdehyde, in agreement with published analyses of other bovine heart plasmalogen (25). Unsaturation content was 1.06 double bond/alk-1-enyl chain, including the enol ether functionality.

Acyl analysis, on the other hand, showed great differences from fraction to fraction. Detailed acyl profiles were recalculated from a weight basis (the flame ionization response of the GC) to a molar basis. The molar amounts then were grouped according to double-bond content to show the distribution of unsaturation fraction by fraction. Table 3 shows this distribution, in units of mol of acyl and alk-1-enyl chain per 50 mol of bovine heart PC (50 mol of PC gives rise to 100 mol of chain). The distribution of the total PC may be compared with the summations from the PC fractions. Concordance is adequate, given the experimental error of the procedures used to generate the data.

The last column of Table 3, average C=C per chain, was calculated as shown in the following example: the first HPLC fraction was 10% of the total lipid, or 5.0 mol out of 50 mol of total lipid. These 5 mol gave rise to 10 mol of chain, of which 5.1 mol were saturated acyl groups (no C=C), 3.9 mol were monoenoic acyl groups (1 C=C), 0.9 mol were alk-1-enyl groups (1.06 C=C) and 0.1 mol were dienoic acyl groups (1 C=C), 0.9 mol was alk-1-enyl groups (1.06 C=C) and 0.1 mol was dienoic acyl groups (2 C=C). These 10 mol of chain therefore averaged 0.5 mol of C=C per mol of chain [(5.1 × 0) + (3.9 × 1) + (0.9 × 1.06) + (0.1 × 2) mol of C=C/10 mol of chain], as indicated in the last column of Table 3. The table also shows that the saturated acyl chains originated mainly in the nonplasmalogen PC, and that the plasmalogen had about double the unsaturation of the nonplasmalogen (1.5 vs 0.8 C=C per chain). Most importantly, the table also shows that the retention times of the fractions increased with the degree of overall unsaturation (0.5-2.5 C=C per chain). The last fraction was so unsaturated, in fact, that 1-hexene was necessary to displace it from the column.

Thus, argentation-HPLC succeeded in fractionating bovine heart PC into several fractions based on overall unsaturation, and not on plasmalogen content alone. Nevertheless, because the plasmalogen was also highly unsaturated in acyl content, it tended to be retained on the column better than the nonplasmalogen. In fact, in the experiment illustrated by Figure 2 and Tables 2 and 3, starting PC, 47% of which was plasmalogen, gave a 49% yield of PC enriched to 72% plasmalogen (combined fractions 3-5).

The influence of the overall unsaturation on the fractionation of bovine heart PC by argentation-HPLC thus confirms the earlier findings by argentation TLC (14,15). Furthermore, the findings concur with a report on the equilibrium of model olefins with silver nitrate surfaces, which found that "despite a possible influence of a large polarization of the carbon-carbon double bond in [alk-1-enyl] ethers, the equilibrium data obtained for these compounds have manifested general features similar to those already recognized for olefins" (26).

Variations on argentation HPLC perhaps could lead to a system more specific for the alk-1-enyl functionality. Mercuric ion, for example, is well known as a coordinator with this functionality, but in aqueous systems leads to hydrolysis and aldehyde formation. Because

water is a mandatory component of mobile phases for the elution of phosphatides from silica-based HPLC columns, mercuration HPLC by the systems used for argentation HPLC would seem to be precluded. A recent report, however, showed that mercuric ion-induced hydrolysis of plasmalogens is inhibited at lower temperatures. Thus, a chloroform solution of plasmalogen-rich lipid in contact with an aqueous solution of mercuric acetate at 0 C transferred Hg II ion into the organic phase; titration of the transferred Hg II ion correlated well with the plasmalogen content (3). This encouraged us to test mercuration HPLC of bovine heart PC at reduced temperatures. The ion exchange column, pretreated with mercuric acetate, was routed through an ice-water bath. Elution of injected bovine heart PC gave an initial fraction composed almost entirely of nonplasmalogen PC. This fraction was followed by 2-acylglycerophosphocholine, the residue of plasmalogen PC. Similar results occurred when mercuric ion was replaced by phenylmercuric ion. Thus, on-column hydrolysis of the plasmalogen by mercuric species could not be suppressed by operating at low temperature. The absence of a purple color when treating eluate from the mercuric columns of the present work with s-diphenylcarbazone indicated that these columns did not suffer leaching of the ions during treatment with the bovine heart PC. Such was not the case, however, with columns treated with Pd II ion; s-diphenylcarbazone detected leaching on introducing the PC to the column.

CONCLUSIONS

Two methods have been demonstrated for isolating PC fractions enriched in plasmalogen. From bovine heart PC containing 47% plasmalogen, RP-HPLC gave a 26% yield of PC enriched to 82% plasmalogen, though the separation of PC fractions did not use the unique functionality of the plasmalogen—the alk-1-enyl group. Argentation HPLC of the same PC gave a 49% yield of PC enriched to 72% plasmalogen. Although the electron-rich alk-1-enyl group offered the potential for selective complexation with silver ion, the total unsaturation content of the PC contributed to the actual separation. Enrichment of the late-eluting fractions in plasmalogen occurred because the plasmalogens were more highly unsaturated than their nonplasmalogen counterparts.

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